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Cells in Response to ECM Signaling

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Eukaryotic chromosomal DNA is folded into topologically independent loop domains by making a periodic attachment at the nuclear matrix. Genomic sequences at these bases of the loops are called matrix attachment regions (MARs), and proteins that specifically bind to MARs may be important for modulating DNA organization and cell function. Whether higher order chromosomal organization is changed due to cell transformation is not well understood. We have previously identified a MAR binding protein of 114kDa from malignant breast carcinomas. The p114 MAR-binding activity was found to be attributed to two separate proteins, poly(ADP-ribose) polymerase (PARP) and SAF-A. The activity was detected in all human breast tumor specimens but was undetectable in normal breast or benign lesions. PARP-depleted breast cancer MDA-MB-231 cells lost the ability to grow in soft agar in an anchorage-independent fashion, showed reduced invasive activity in vitro, and exhibited changes in cell morphology on Matrigel. We compared gene expression profiles of control MDA-MB-231 cells with PARP-deleted MDA-MB-231 cells and found many genes related to DNA/RNA binding, cell cycle regulation, chromosome assembly and redox regulation to be up-or down-regulated upon PARP deletion. Our data suggest that studies on the role of MAR-binding proteins will advance our knowledge on the mechanisms underlying breast tumor progression				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction	4
Body	5
Key Research Accomplishments.....	13
Reportable Outcomes.....	13
Conclusions.....	13
References	14

INTRODUCTION

Specialized AT-rich sequences with high base unpairing propensity are found in genomic DNA at a frequency of about one every 30-50 kilobase pairs. Such base unpairing regions (BURs) are represented by a special DNA context where one strand consists of well mixed As, Ts, and Cs, but no Gs (ATC sequences). BURs are less than 200bp and enriched in such ATC sequence stretches. BURs are also found as key structural elements of MARs (reviewed in Kohwi-Shigematsu and Kohwi, 1997). Recent studies have shown that certain MARs play roles in cell-type specific transcription and generate long-range chromatin accessibility to transcription factors (Forrester *et al.*, 1994, and Jenuwein *et al.*, 1997). Also, certain MARs are responsible for demethylation and acetylation across the chromatin domain (Fernandez *et al.*, 2001., Kirillov *et al.*, 1996., and Lichtenstein *et al.*, 1994.). Recently, our host laboratory reported the biological function of a cell-type specific MAR-binding protein SATB1 based on the knockout mouse analyses (Alvarez *et al.*, 2000). SATB1 coordinates temporal and spatial expression of a large number of genes during T cell development. SATB1 *in vivo* binding sequences are located at the bases of chromatin loop domains (deBelle *et al.*, 1998). These studies indicate that MARs and MAR-binding proteins have important biological function presumably by regulating higher order chromatin structures.

The BUR-binding activity due to p114 protein was previously identified in human breast carcinoma specimens but not in non-malignant cells (Yanagisawa *et al.*, 1996). The BUR binding activity of this protein was inversely proportional to the differentiation status of the breast tumors. Advanced tumors of mostly infiltration ductal carcinoma from patients who have extensive metastases display a significantly higher level of p114 BUR-binding activity in comparison to tumors of mostly intraductal carcinoma. We found this p114 protein to be poly ADP-ribose polymerase (PARP).

PARP is a nuclear enzyme known to synthesize ADP-ribose polymers from NAD, and is activated in response to DNA strand breaks (reviewed in D'amours *et al.*, 1999). It was unexpected that PARP can recognize primary sequences like BUR besides DNA ends and nicks. My host laboratory has also reported that the DNA-binding subunit of the DNA-dependent protein kinase (DNA-PK), Ku autoantigen (Ku70/86) and PARP form a protein complex and bound to a BUR affinity column. These proteins have roles in DNA repair, recombination, DNA replication, and transcription (Galande and Kohwi-Shigematsu, 1999). PARP and Ku70/86 individually bind to BURs.

However, their affinity to BURs is synergistically enhanced when they make a protein complex. ADP-ribosylation of the nuclear extract abrogated the BUR-binding activity of this complex (Galande and Kohwi-Shigematsu, 1999).

In addition to PARP, the expression of purified 20 kDa proteins by BUR affinity column from human breast cancer cells, which were identified as HMG I (High Mobility Group I) and HMG Y, are also correlated with the metastasis status of breast cancer cells (Liu *et al.* 1999).

The greatly increased levels of MAR binding proteins such as PARP and HMG I(Y) may change the three-dimensional organization of chromatin through their BUR binding activity and thereby alter regulation of a large number of genes. Through the research with BUR binding proteins, we will also examine the link between signal transduction pathway and chromatin structure in breast cancer cells. The increase in expression of MAR-binding proteins would be critical for the onset or maintenance of the malignant phenotype of breast cancer cells. Through studying the function of MAR-binding proteins in breast cancer, we aim to obtain an important insight into the fundamental mechanism underlying breast cancer development and/or maintenance. Our work may also provide a clue for breast cancer therapy in the future.

BODY

Specific Aim 1. To establish the correlation between p114 MAR-binding activity and the alteration of cell shape associated with either normal or malignant phenotype. To determine whether p114 MAR-binding activity is critical for the onset and/or maintenance of the malignant phenotype.

Progress 1. We established the additional five stable transformants, in which PARP is depleted by expressing antisense PARP RNA using aggressive human breast cancer cells, MDA-MB-231. Very low levels of PARP expression for each transformants was confirmed by western analysis. These transformants were confirmed by my hands to show the following phenotypes.

1. PARP depleted cells lost their abilities to grow in soft agar in an anchorage-independent fashion.
2. The proliferation rates of PARP depleted cells and its host cells, MDA-MB-231 are the same in plastic culture dishes. Therefore, a high level of PARP expression in breast cancer cells is not directly linked to a high rate of proliferation.
3. PARP-depleted cells on matrigel (the ECM derived from the Engelbreth-Holm-Swarm murine sarcoma (EHS matrix)), however, proliferate much more slowly than host MDA-MB-231 cells. Also, PARP-depleted cells changed their cell morphology on matrigel.

Progress 2. Previously we found that BUR binding activity of p114 is comprised from two different proteins; one is PARP and the other is SAF-A (scaffold associated factor A, as known as one of BUR-binding proteins).

This fact was found by southwestern analysis and immunoprecipitation with anti-PARP antibody. We examined the expression level of SAF-A in breast tumor and its normal counterparts (Fig.1). Our previous results have already indicated that PARP is up-regulated in breast tumors as shown in Figure 1A. The expression of SAF-A is also up-regulated in breast tumor specimen compared with its normal counterpart (Fig 1B). It strongly suggests that the activities of multiple MAR (BUR) binding proteins may be critical determinants of breast tumorigenesis.

Specific Aim 2. To identify genomic sequences bound to p114 in vivo in T4-2 cells grown in EHS matrix. The association of these sequences with nuclear matrix prepared from T4-2 cells either grown in EHS matrix (tumor phenotype) or treated with α_1 -integrin antibody (non-malignant phenotype) will be compared.

Instead of T4-2, we decided to change the type of the cells studied in Specific Aim 2 and 3 to MDA-MB-231 cells. The reason for this is that MDA-MB-231 cells are more aggressive than T4-2 cells and we already learned that, upon PARP ablation, they lost anchorage independent growth and reduced their invasiveness. We have already established several PARP-depleted cell lines using MDA-MB-231 cells, and they are ready for direct comparison to host cells. Apparent alterations in the cell morphology on matrigel, where the PARP depleted cells form a spheroidal clones (mammospere) (AS12) or slow growth rate (AS7) which is not observed in aggressive MDA-MB-231 breast cancer cells, strongly suggest that there must be a major difference in gene expression before and after PARP ablation.

Progress 1. We preliminarily identified 50 genomic DNA fragments bound to PARP in vivo in MDA-MB-231 cells grown in plastic culture plates without ECM signals at first. The cross-linked genomic DNAs were immunoprecipitated with anti-PARP antibody (summarized briefly in Fig.2) or with preimmune mouse serum. Ligation mediated PCR showed the products exclusively from the anti-PARP antibody precipitated chromatin fraction and ranged in size of 100-1000bp (Fig.2, upper panel, lane 1). Ten DNA fragments selected from transformed colonies conferred binding to GST-fused PARP proteins using gel shift assay under the condition previously employed by Galande and Kohwi-Shigematsu, 1999 (two representative cases of gel-shift data are shown in the lower panel, Fig. 2). However, in addition to linear DNA as templates, we will further check this fact with closed circle DNAs as templates as exactly described in the above-mentioned paper. We will be sequencing the entire length of inserts for all ten clones shortly.

Specific Aim 3. To identify specific gene nearby the genomic sequences bound to p114. To determine whether the expression of these specific genes is regulated by p114 MAR binding activity altered by ECM signaling.

Progress 1. First, in order to get an insight as to what types of genes are altered their expression levels due to PARP ablation, we used human microarray (LBL supplied) that contains 20,000 genes. Table 1 and Table 2 showed the combined results for increased and decreased gene expression profiles, respectively. Two PARP-depleted cell lines (AS7 and AS12) were compared with their host cell line MDA-MB-231 without ECM signals at first. The fold difference in the tables is a log transform of the ratio in intensity of each median on microarray. The ablation of PARP seemed to cause changes in redox balance inside cells since several redox related gene (such as peroxiredoxin, hydratase, etc.) were upregulated in PARP-depleted cell lines. The cytoskeletal protein such as fibronectin is remarkably up-regulated by PARP ablation. While the genes related with

DNA repair, cell cycle regulation, DNA/RNA binding and chromosome assembly (hPMS1, CDC27, ERCC3, SMC4 like, etc.) were down-regulated. Some of these were confirmed with RT-PCR analysis as shown in Figure 3.

Immediate future plan: We will also compare the gene expression profiles between PARP-depleted and wild type host cells grown on matrigel to examine the effect of ECM signals as the next step.

In parallel, we will determine genes that are located nearby each of the PARP-bound sequences in vivo described above. We will examine if there is any difference in the expression levels of these particular genes between host MDA-MB-231 cells and PARP-depleted cell lines on plastic culture and on matrigel.

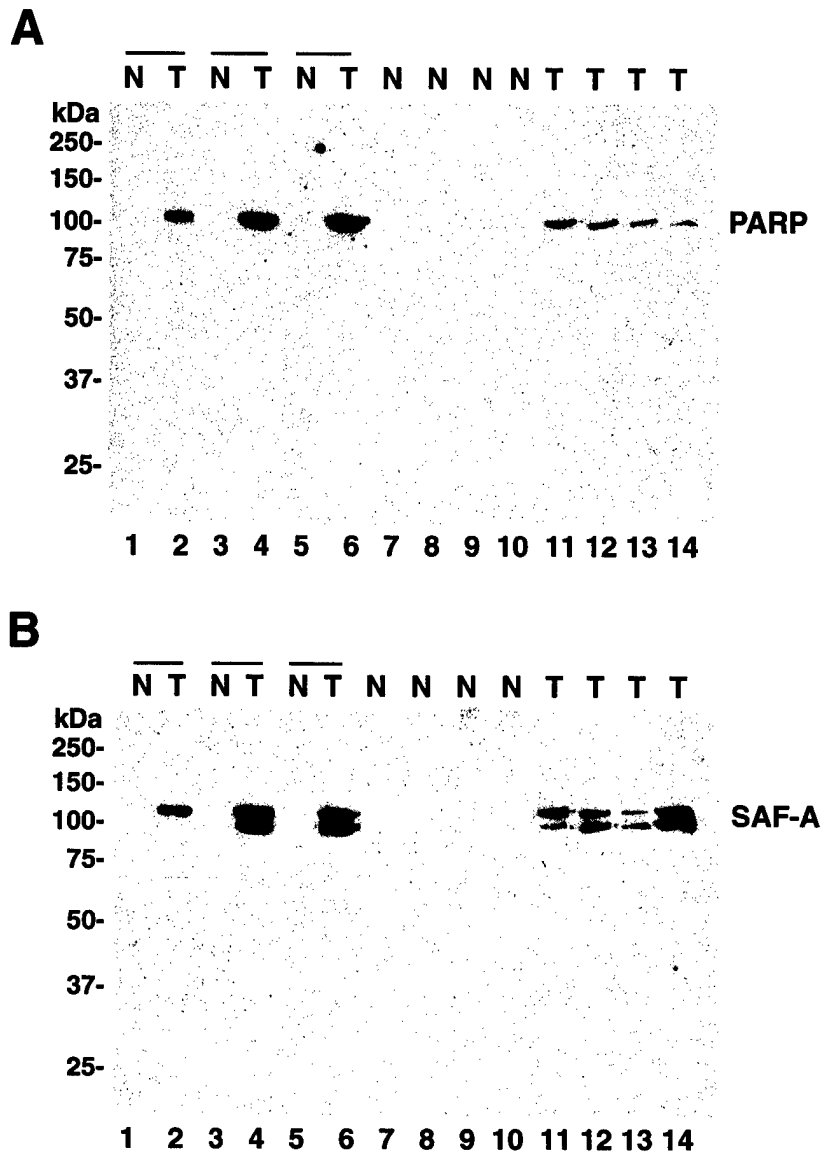


Fig.1 PARP and SAF-A are detected only in breast carcinoma specimens but not in normal breast tissues.

Immunoblot analysis of PARP (A) and SAF-A (B) expression in different normal breast tissues and non-malignant breast lesions (N) and breast carcinoma specimens (T) was performed. Fifteen microgram of proteins in tissue extracts prepared from three cases of ductal carcinomas (lanes 2, 4 and 6) and their adjacent normal tissues (lanes 1, 3 and 5) were loaded on a 10% SDS-PAGE. Donor matched samples are indicated by a line on top of the lanes. Additionally, four non-matched breast tissue samples from each of normal breast (lane 7 and 8), breast with fibrocystic change (lane 9), and with atypical hyperplasia (lane 10) and invasive breast carcinomas (lanes 11-14) were loaded. After electrophoresis, the proteins were transferred onto a PVDF membrane and probed with polyclonal anti-PARP (H250, Santa Cruz Biotechnology) or anti-SAF-A (kind gift from Dr. Frank Fackelmayer). Positions of the molecular weight markers are indicated on the left in kDa. The positions of signals corresponding to PARP and SAF-A are indicated on the right.

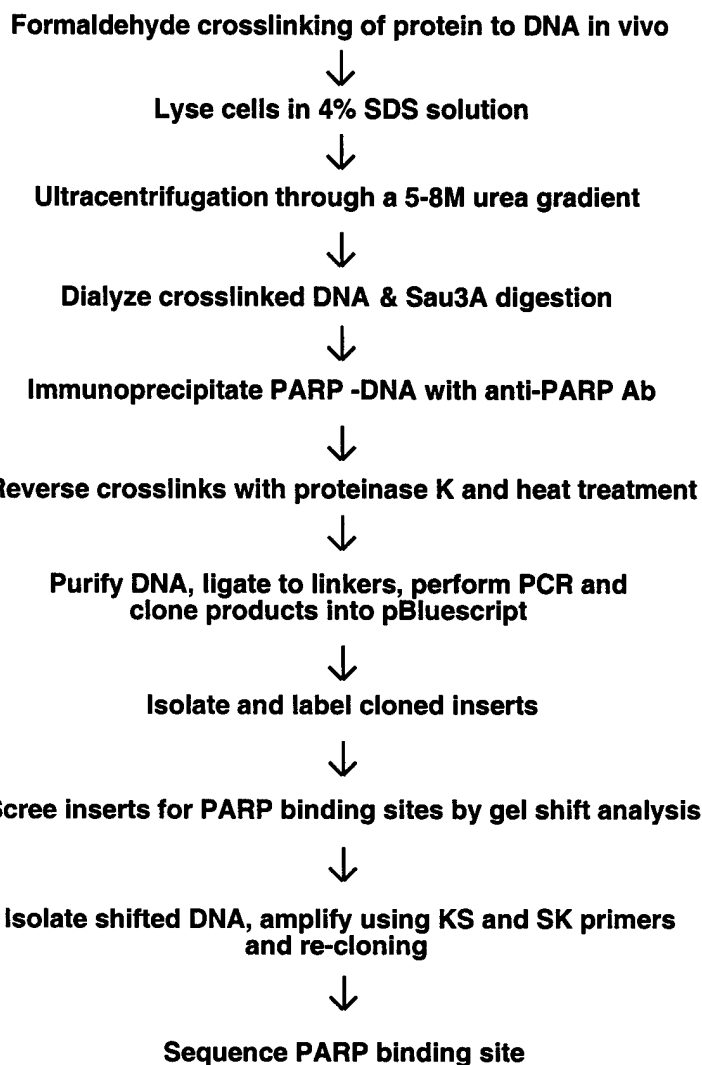
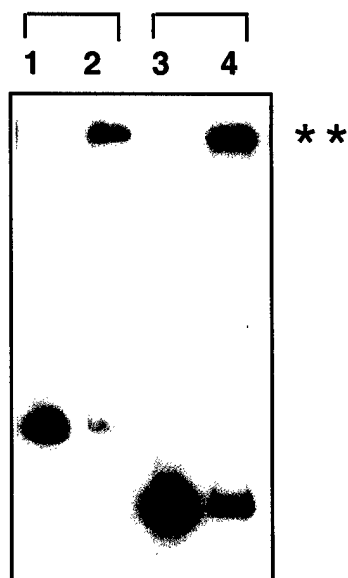
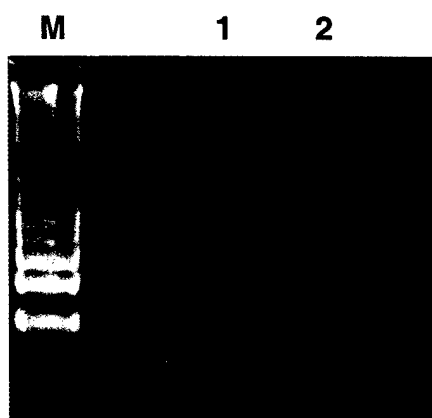


Fig.2. PARP binds to genomic DNAs in vivo.

In vivo cross-linked genomic DNA pools from MDA-MB-231 cell lines were immunoprecipitated with monoclonal anti-PARP antibody (4C10-5, BD Pharmingen) and mouse preimmune IgG1 and IgG2 (as negative control). The upper panel shows PCR amplifications of immunoprecipitated DNA after linker after 17 cycles. PCR products from anti-PARP immunoprecipitated DNA (lane 1) and preimmune (lane 2) were examined by 1.2% agarose gel electrophoresis followed by staining with ethidium bromide. Lane M, 1kb molecular size marker. The lower panel shows a gel mobility shift assay with anti-PARP immunoprecipitated DNA. PCR products shown in lane 1 in upper panel were isolated from the gel and cloned into pBluescript. Insert DNAs in different size ranges were tested for PARP binding activity. These are one of cases we tested. Lane 2 and 4 showed shifted band by PARP binding (** mark in right side). Lane 1 and 3 are free probe of each.

Table 1. Differential expression profiles of increased genes between MDA-MB-231 cells and PARP-deleted MDA-MB-231 cells.

Fold increase in expression in PARP-deleted cells relative to that in wild-type cells.

Increased genes	ID	Acc. No.	Name	Symbol	Locus	Log fold
ESTs	687297	AA235224	cDNA FLJ11245		1	8.1
	796531	AA463824	EST		6	4.5
	666707	AA234027	EST			4
	840726	AA487846	EST			3.2
	782547	AA431796	EST		11	4.3
	144855	R78533	EST		9	3.3
	220069	H85437	EST		15	6.6
	767136	AA424614	Hypothetical protein MGC2668			3.1
	742094	AA405773	Hypothetical proteinFLJ20950		14	2.1
	144762	R77213	cDNA FLJ20848		16	4
	827132	AA521232	HSPC022 protein	HSPC022	22	3.8
	768481	AA425032	Hypothalamus protein HT011	HT011	10	2.8
	435024	AA700048	Gene from NF/meningioma	PK1.3	22q12	2.3
	139009	R62662	Fibronectin	FN1	2q34	10.3
	51814	H24099	Cystatin B (Stefin B)	CSTB	21q22.3	6
Cellular structure	810612	AA464731	S100 calcium binding protein A11 (calgizzarin)	S100A11	1q21	5.2
	756595	AA444051	S100 calcium binding protein A10 (annexin II)	S100 A10	1q21	4
	743229	AA400329	Neurofilament3	NEF3	8p21	4
	786072	AA448758	Myocin1C	MYO1C	15q21	3.2
	309893	W23937	Nuclear receptor subfamily 4 (steroid hormone receptor)	NR4A1	12q13	6.2
Receptors	565379	AA136336	Growth factor receptor-bound protein	GRB10	7p12-p11.2	11.3
	811899	AA456265	Proteinase activated receptor-2			3.8
	436155	AA703250	PRP4/STK/WD splicing factor	HPRP4P	9	4
DNA/RNA binding	292357	N80989	Ring finger protein 9	RNF9	6p21.3	3.2
	810734	AA480820	Polymerase (DNA-directed), delta 4	POLD4	11	4
	81417	T60223	Ribonuclease	RNASE4	14	1.7
	343443	W67200	RNA-binding protein gene	RBPM5	8p12-p11	2.6
	469412	AA026918	Fumarate hydratase	FH	1q42.1	7.4
Redox/Metabolite	809894	AA464329	Acetyl-CoA synthetase	LOC55902	20	4.4
		AA873159	Apolipoprotein C1	APOC1	19q13.2	4.1
	866882	AA679352	Farnesyl-diphosphate farnesyltransferase 1	FDFT1	8p23.1	3.5
	795198	AA453577	Zinc/Iron regulated transporter	ZIRT1	1q21	3.2
	292519	N91311	Peroxiredoxin 5	PRDX5	11	4
Anti-apoptosis	50117	H16957	Glyceraldehyde-3-phosphate dehydrogenase	GAPD	12p13	4.3
	810331	AA464217	Quiescin Q6 (growth factor BPGF-1)	QSCN6	1q24	4.4
	781222	AA446223	TGFBI-induced anti-apoptotic factor	TIAF1	17	3.4
	79448	T60019	Development and differentiation enhancing factor	DDEF1	8q24.1	2.8
	810039	AA465001	Defender against cell death 1	DAD1	14q11	3
Miscellaneous	210697	H66884	EHM2 gene	EHM2	9	2.4
	77533	T58845	Inositol polyphosphate-5-phosphatase	INPP5A	10q26.3	3.4
	48801	H14843	Popeye protein 3	POP3	6	3
	809494	AA456183	CD151 antigen	CD151	11p15.5	3.5
	824527	AA491087	Guanylate kinase 1	GUK1	1q32-q41	3.5
	756533	AA481383	Basigin (OK blood group)	BSG	19p13.3	3.1
	435351	AA700739	BUP protein	BUP	10	4.6
	307337	W21055	KIAA0758	KIAA0758	6	3.5
	754046	AA479062	ITBA2	DXS9879E	Xq28	12.8

Table 2. Differential expression profiles of decreased genes between MDA-MB-231 cells and PARP-deleted MDA-MB-231 cells.

Fold decrease in expression in PARP-deleted cells relative to that in wild-type cells.

Decreased genes	ID	Acc.No.	Name	Symbol	Locus	Log fold
EST	505810	AA152351	EST		7	3.8
	357117	W93520	Hypothetical protein	FLJ13194	3	4.6
	250822	H96095	Hypothetical protein	FLJ14600		3.3
	380884	AA058576	EST		1	2.4
	277707	N49581	EST		8	2.2
	197056	R92801	EST		3	2.2
	745157	AA478894	cDNA DKFZp434K2172		7	2.4
	842896	AA489276	Hypothetical protein			3.4
	486179	AA043612	cDNA FLJ10205		16	2.5
	565734	AA135984	EST			2.4
	838616	AA457077	FLJ20093		13	3.3
	37653	R61345	cDNA DKFZp564D1164		15	2.4
DNA/RNA binding	684644	AA251581	Zinc finger protein 198	ZNF198	13q11	2
	486356	AA043743	Zinc finger protein 83 (HPF1)	ZNF83	19q13.3	2
	786592	AA452256	Zinc finger protein 265	ZNF265	1p31	3.1
	131791	R24606	Splicing factor, arginine/serine-rich 11	SFRS11	1p21-p34	2.6
	745503	AA625995	Zinc finger protein 9 (CNBP)	ZNF9	3q13.3-q242.5	2.5
	78695	T61866	RAN binding protein 7	RANBP7	13	4.1
	51666	H20908	DNA repair helicase (ERCC3)	ERCC3	2q21	2
	133136	R28400	DEK oncogene (DNA binding)	DEK	6p23	3
	345069	W76339	Erythroid-derived transcription factor	NFE2L3	7p15	3.8
	451504	AA707321	pinin, Nuclear protein SDK3	PNN	14	2.6
	278630	N99195	Helix destabilizing protein (hn protein)	FBRNP	10	4
	897823	AA598578	Heterogeneous nuclear ribonucleoprotein D	HNRPD	4	3.7
	293635	N94166	Heterogeneous nuclear ribonucleoprotein A2/B1	HNRPA2B1	7p15	2.5
Cell cycle	825726	AA505166	Postmeiotic segregation protein	PMS1	2q31.1	3.6
	781047	AA430092	BUB1 mitotic checkpoint protein kinase	BUB1	2q14	2.7
	504396	AA142869	Cell division cycle 27	CDC27	17q12-q23.2	3.3
Chromatin	745360	AA625662	Histone Acetyltransferase 1	HAT1	2q31.2	2.8
	786504	AA452317	chromosomal protein CAPC homolog	SMC4L1	3q26.1	3.4
	45941	B09055	DNA-methyltransferase 1	DNMT1	19p13.2	2.1
Signal pathway	769003	AA425091	Serum/Glucocorticoid regulated kinase	SGK	6q23	4.3
	1325605	AA875888	Neuron-specific protein	NSG1	4	2.4
	714106	AA284669	Fibrinogen activator, urokinase	PLAU	10q24	3.6
	487141	AA045428	Abl-interactor 12 (SH3 containing protein)	AIP-1	2	2.7
Miscellaneous	526184	AA079416	GTP-binding protein (RAB21)	KIAA0118	12	3.2
	134419	R31723	Endomembrane protein emp70	LOC56889	10	3.7
	700299	AA290914	WASP interacting protein (WIP)	WASPIP	2p23.3	3.8
	345601	W76395	Melanoma associated gene	D2S448	2pter-p25.1	3.3
	859185	AA666316	Mitochondrial carrier	SLC25A12	2q24	2.5
	788242	AA454083	CGI32	LOC51076	10	2.6
	730633	AA411969	Cisplatin resistance associated gene	LUC7A	17	2.8
	289939	N77149	PIBF1 gene	PIBF1	13	2.7
	754108	AA479203	KIAA1109	KIAA1109	4	3
	595162	AA174030	Axon guidance receptor	ROBO1	3p12	2.6
	290378	N80294	Podocalyxin	PODXL	7q32	3.6
	726571	AA398129	endomembrane protein, emp70	LOC56889	10	3.32
	1455394	AA865265	cytochrome c	HCS	7	3.4
	969914	AA772816	Acidic 82kDa protein	HSU15552	1	2.4
	753198	AA406456	C-Myc target JPO1	JPO1	2	3.3

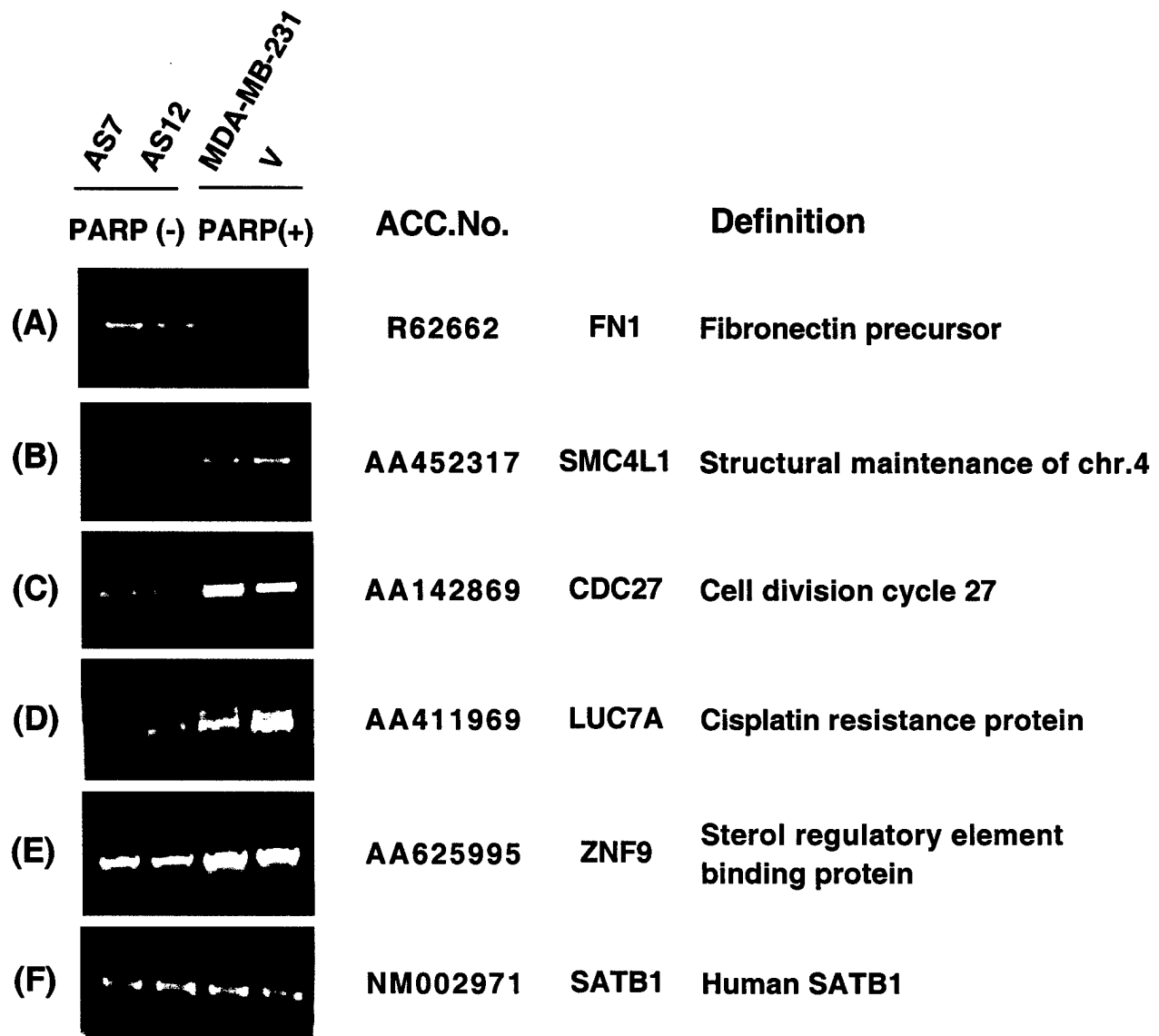


Fig.3. Validation of microarray data by semiquantitative RT-PCR. RT-PCR was used to amplify fragments of FN1 (A), SMC4L1 (B), CDC27 (C), LUC7A (D), ZNF9 (E), and hSATB1 (F) from RNAs of PARP-depleted MDA-MB-231 cell line (PARP(-): AS7 and AS12) and their control cell lines (PARP(+): MDA-MB-231 and vector transformed MDA-MB-231 cells). FN1 gene (A) is used for an example of increased gene expressions in PARP depleted cells. The rest (B-E) are examples for decreased genes in PARP depleted cells. The hSATB1 amplified from each sample shows similar loading in each lane (F).

KEY RESEARCH ACCOMPLISHMENTS

1. SAF-A is upregulated in breast tumor specimens in addition to HMGI(Y) and PARP. This shows that multiple BUR-binding proteins could be key players in the maintenance of the aggressive phenotype of breast malignancy.
2. The apparent differences in cell morphology and the growth rate between host MDA-MB-231 cells and PARP-depleted counterparts on EHS matrix suggest that PARP has a role in the way that cells respond to ECM signaling.
3. A total of ten genomic DNA fragments bound to PARP *in vivo* were identified in MDA-MB-231 cells.
4. The human gene microarray analysis showed 47 increased and 50 decreased gene profiles at more than 2 fold differences in log intensity between the two fluorescence labelled probes prepared from PARP depleted and MDA-MB-231 cell lines.
5. PARP ablation resulted in downregulation of genes involved in regulation of cell cycle progression or mitosis, DNA repair, or chromosomal processing. It also upregulates genes that encode survival factors, redox regulators, extracellular matrix or cytoskeletal proteins.

REPORTABLE OUTCOMES

1. Establishment PARP depleted MDA-MB-231 breast cancer cell lines.
2. Additional publications in this period
 - a) Galande S. and Kohwi-Shigematsu T. Linking chromatin architecture to cellular phenotype: BUR-binding proteins in cancer. *J Cell Biochem. Suppl* 35:36-45. (2000)
 - b) Galande S. and Kohwi-Shigematsu T. Caught in the act: binding of Ku and PARP to MARs reveals novel aspects of their functional interaction. *Crit Rev Eukaryot Gene Expr.* 10(1):63-72 (2000).

CONCLUSIONS

We established several PARP depleted MDA-MB-231 cell lines to compare their phenotype and gene expression against their host cell line MDA-MB-231 cell. They clearly showed differences in growth in soft agar, morphology on EHS matrix, and growth rate on EHS matrix. In addition, we showed significant differences in the gene expression profiles between PARP-depleted and wild type MDA-MB-231 cells on plastic culture dishes.

The ECM does not only provide a support for cells *in vivo* but its components also act as ligands that interact with cells via cell surface

molecules. The tissue matrix system is also believed to form a structural and functional connection between the cell periphery and DNA, establishing a mechanical signaling pathway to transmit signals from the cell's exterior to nuclear DNA. From the results obtained, it is likely that PARP has a role in linking ECM signaling to nuclear function such as gene regulation, presumably by modulating chromatin structures through BURs. Such PARP function is expected to be important for breast cancer development or maintenance. Our remained task will be to characterize the genomic sequences that are bound by PARP in vivo and expression profiles of genes nearby in the presence of absence of PARP when cells are grown on matrigel or plastic dishes. The data will give important information regarding function of BUR-binding proteins for onset and/or maintenance of breast cancer malignancies.

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